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2. Patent application number *(The Patent Office will fill in this part)* 0217963.8 02 AUG 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
CYCLOPS GENOME SCIENCES LIMITED
66 CHURCH ROAD
WEST KIRBY
WIRRAL L48 ORP

Patents ADP number *(if you know it)* 76518620002

If the applicant is a corporate body, give the country/state of its incorporation UK

4. Title of the invention PURIFICATION OF NUCLEIC ACIDS

5. Name of your agent *(if you have one)* SEE ~~ABOVE~~
Page White + Farrer
54 Doughty St
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WC1N 2LS

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

51/77
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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application.

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Purification of Nucleic Acids

Summary

Described is a method for purifiying DNA and RNA from a sample, such as a biological sample or a clinical sample including cells, blood, serum and plasma. Advantageously, when the analyte is RNA, it is protected from degradation by chemical modification of the 2'-OH groups. The chemically protected RNA can be consequently deprotected using an organic primary amine which have been found to be particularly suited for this purpose. For both DNA and RNA purification, the method provides a means to lyse cells and virus particles and remove proteins. Elution from the solid phase hydroxylapatite purification matrix is effected using a metal ion chelator such as EGTA.

Background

Various methods are known for the purification of nucleic acids such as (i) the use of a salt chaotrope and silica surfaces, (ii) a phenol based extraction, and (iii) a chaotrope and precipitation of the nucleic acid. Methods for the purification of nucleic acids have been extensively described (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH).

One of the drawbacks of these methods is that the purified nucleic acids are contaminated with proteins and other biomolecules that lead to inhibition of downstream enzymatic applications and possible cleavage of the nucleic acid. Many of the methods are also time consuming and result in loss of a significant proportion of the desired nucleic acid analyte. Indeed one of the downsides of obtaining higher purity nucleic acid is that the overall yield of the nucleic acid is reduced. We have developed a method that provides nucleic acids of high purity and yield, but also in the case of RNA, are chemically protected from degradation.

Deprotection of Nucleic Acids

Part of the invention relates to methods to remove acyl groups, in particular acetyl groups from acetylated molecules, in particular biomolecules such as DNA and RNA. It is well known that many if not all methods that have been described to remove acetyl groups from acetylated compounds can lead to non-desired products along with the desired non-

acetylated product. This is because the acetylated nucleic acid may contain groups other than the acetyl group such as the phosphodiester backbone that are cleaved by the deprotecting compound. In this case the deprotecting compound is defined as a material that can remove an acyl group and in particular acetyl groups from an oxygen (acetyl) or nitrogen (acetamide) to restore the original hydroxyl or amide group respectively with only limited degradation of the nucleic acid itself.

The ease with which the acyl group can be removed from the acylated compound depends on a number of parameters that have been described extensively in « Protecting Groups in Organic Synthesis » Greene and Wuts, 2nd Edition, Wiley Interscience. In general, acyl groups are removed more easily from oxygen than from nitrogen. Another major factor is the group attached to the carbonyl, for example the formyl group (-CO-H) is simply removed at pH 9 and above, whilst the acetyl group (-CO-CH3) requires much harsher conditions to remove it. Longer chain lengths such as propanoyl (-CO-CH2-CH3) and butanoyl (-CO-CH2-CH2-CH3) are even more difficult to remove whilst substituted acyl groups such as methoxyacetyl (-CO-CH2-O-CH3), chloroacetyl (-CO-CH2Cl) or trifluoracetyl (-CO-CF3) are much more readily removed than the unsubstituted acetyl. However, acetyl remains the predominately used acyl protecting group throughout industry, in part because it can be readily added to compounds from cheap and easily used reagents such acetic anhydride, acetyl chloride and acetyl-imidazole. Furthermore, it has been found than when used in the presence of aqueous solutions such as blood, acetic anhydride is less liable to hydrolysis and therefore inactivation than reagents such as methoxyacetic anhydride or chloroacetic anhydride. Therefore it is necessary to use acylating reagents that are not so unstable in aqueous solutions that they hydrolyse before they can modify the nucleic acid, whilst modifying the nucleic acid with chemical groups that can be removed without leading to nucleic acid destruction. This is particularly problematic for RNA which is readily degraded by the types of alkalis that are efficient at removing the acyl groups. Methods of using such alkalis for the deprotection of the 2'-OH groups of RNA have been described elsewhere (WO/01/94626, WO/00/75302).

Numerous methods have been described to remove acetyl groups from acylated compounds including enzymatic, electrolytic and chemical means. Whilst enzymes such as esterases and lipases have found widespread use due to their mild reaction conditions, they are expensive,

are often contaminated with non desired proteins or compounds and require careful pre-selection to find an appropriate activity. They are also sensitive to the charge on the acylated compound so that strongly charged molecules such as nucleic acids may not be good substrates.

Whilst many chemical deprotecting methods are also known for removing acetyl groups (Protecting Groups in Organic Synthesis, Greene and Wuts, 2nd Edition, Wiley Interscience) most if not all involve either a base or acid, conditions that are likely to lead to extensive cleavage of the desired RNA during deprotection.

Methods for protecting RNA by chemical modification have been extensively described in Patent application numbers WO/01/94626 and WO/00/75302. Fully acetylated RNA is protected from degradation from nucleases, however it is not capable of serving as a reverse transcription template and neither cannot it hybridise. It is therefore important, after the RNA has been protected to be able to remove the acetyl groups in order to allow efficient reverse transcription, hybridisation and to serve as a template for protein synthesis. Methods to remove acetyl groups from RNA have been described in patent application numbers WO/01/94626, WO/00/75302. These methods include the use of potassium cyanide, Hunigs Base, dimethylethylenediamine, sodium hydroxide and ammonium hydroxide.

Unfortunately, RNA is extremely sensitive to the presence of alkali and RNA chain cleavage occurs after the acetyl group has been removed from the 2'-OH position. It is therefore necessary to use a pre-calibrated amount of alkali sufficient to remove the acetyl groups from the RNA but not so much that leads to significant subsequent RNA cleavage. Whilst this has been achieved by the use of mixtures of either sodium hydroxide or ammonium hydroxide with alcohol, some RNA chain cleavage is an inevitable result of acetyl deprotection using these methods (discosed in patent application; WO/01/94626, WO/00/75302). Many other methods and chemicals have been tested for their activity to remove acetyl groups from RNA without leading to its cleavage including sodium hydrogen carbonate, sodium carbonate, potassium carbonate, potasium hydroxide, triethylamine, guanidine, hydrazine and HCl. These compounds either had no activity or lead to some degree of RNA chain cleavage and are therefore not the preferred method for deprotection of RNA although they are useful for the removal of acyl groups from DNA oligonucleotides

and polynucleotides when the acyl group is either attached to the 5'-OH, the 3'-OH or the nucleobases. DNA is much less sensitive than RNA to being cleaved by alkalis. For example DNA can be incubated in 1M NaOH for one hour with no detectable degradation, conditions which would reduce RNA to monomers within 5 minutes. Others methods that have been described in the literature as either mild or 'ultra-mild' deprotection methods (Glen Research, USA), are completely inappropriate for RNA because they lead to extensive chain degradation. Furthermore, both methylamine and ammonium hydroxide which are widely used for deprotection are toxic and dangerous to work with and very strong smelling. They are not therefore suited to daily laboratory settings and must be used in a fume hood.

One published method (Boal et al., Nucleic Acids Res. (1996) 15:3115-3117) describes a method to deprotect benzoyl, isobutyrl or isopropoxyacetyl nucleobases on oligonucleotides following synthesis, employing gaseous ammonia at 10 bar pressure or methylamine at 2.5 bar in a pressured device. It is not expected that pressurised use of gases will find widespread acceptance in laboratories.

It has been found that either modified RNA or RNA that has been immobilised on a solid phase such as charged nylon (Hybond N+, Amersham Biosciences, UK) and then treated with an alkali such as sodium hydroxide or ammonium hydroxide is more protected from subsequent alkali degradation than similar RNA in solution. This may be because the immobilised modified RNA or RNA has limited torsional freedom, that is the 2'-hydroxyl group in the presence of alkali and a solid phase cannot subsequently attack and cleave the 3'-phosphodiester bond as would occur in solution. The cleavage effect of the alkali on the RNA is therefore reduced. Whilst this method is useful for deprotecting modified RNA for hybridisation purposes such as northern blotting, it has limited application for deprotecting modified RNA for reverse transcription or transcription mediated amplification (TMA) because polymerases such as reverse transcriptases cannot effectively copy immobilised RNA. Methods for deprotecting modified RNA on solid phases have been set out in Patent application numbers (WO/01/94626, WO/00/75302).

Other solid phases that provide only a temporary reversible binding of the modified RNA to the surface such as silica (Qiaex II particles (Qiagen, Germany)) were found not to be useful for deprotection of RNA with aqueous alkalis because the RNA is eluted from the surface

into the alkali during deprotection leading to its cleavage at normal rates. Other solid phases such as hydroxylapatite, although they bind RNA even in the presence of alkali, did not provide the level of protection from cleavage provided by charged nylon.

However, when acetyl modified RNA is bound onto a solid phase such as silica or hydroxylapatite it can be effectively deprotected without significant cleavage using dry gaseous ammonia. In this example, the solid phase may not be reducing the amount of cleavage, rather it may simply present the modified RNA to the ammonia such that a substantial proportion of the acetyl groups are readily accesible. By contrast, a precipitated or spin-dried pellet of RNA is less readily deprotected by ammonia gas possibly because of the difficulty of the ammonia entering the pellet and contacting all the acetyl groups. It is important that the ammonia vapour is dry because it has been found that the presence of water leads to RNA cleavage.

Pressurised bottled ammonia is a suitable dry source or alternatively, ammonia can be conveniently distilled from a solution of 28% ammonium hydroxide. In the latter example, it is important to pass the ammonia vapour over at least one surface to condense any water vapour that may have been carried over with the ammonia from the ammonium hydroxide solution. The dried ammonia can then be passed into a flask containing one or more tubes bearing the modified RNA bound onto beads. Deprotection times vary from a few minutes to one hour, depending on (i) the concentration of ammonia, (ii) the temperature, and (iii) the amount of RNA exposed to the ammonia in the particle pellet. It has been found that dense pellets of silica or hydroxylapatite bearing the modified RNA are deprotected more slowly than diffuse pellets of particles. Controlling the pellet size can be difficult and therefore it can be difficult to judge the necessary deprotection time. Deprotected RNA can be simply washed to remove traces of the by product ammonium acetate and eluted for downstream applications. Unfortunately this method is somewhat cumbersome and unpredictable for routine use and involves working in a high performance chemical fume hood.

An improved method of deprotection has been developed that does not involve gaseous materials. Although the use of methylamine (CH_3-NH_2) is known for the removal of acyl groups from acylated compounds, it is a dangerous, gaseous and toxic chemical to use. One of the problems is that methylamine is its low boiling point (-6°C) and a high vapour

pressure (2250mm Hg at 20°C) so that evaporation rates are very high indeed. Similarly ammonia boils at -33°C and has a high vapour pressure making its use dangerous. Both ammonia and methylamine are gases at room temperature and pressure. This preferred method involves the use of primary amine compounds with vapour pressure below 2000mm Hg, more preferably below 1000mmHg, even more preferably below 500mm Hg and most preferably below 200mm Hg at 20°C. This preferred method involves the use of primary amine compounds with boiling points above 0°C, more preferably above 25°C, even more preferably above 50°C and most preferably above 100°C at 1 atmosphere of pressure.

Long chain primary amine compounds such as octylamine have boiling points above 100°C, however, the rate of deprotection with these compounds is expected to be slower than for shorter chain primary amines such as butylamine because of steric hinderance between the nucleic acid and the bulky long chain amine. There is therefore a trade-off between the rate of deprotection of the primary amine and it's boiling point/ volatility. However, advantageously, compounds that can extensively hydrogen bond such ethanolamine and ethylenediamine are not only relatively small molecules, thereby reducing steric hinderance, they also have boiling points above 100°C. Therefore primary amine compounds that can also hydrogen bond extensively are both useful because they deprotect quickly but also because they have significantly lower vapour pressures so they are easy to handle.

Advantageously it has been found that deprotection can be carried out whilst the RNA is immobilised on a solid support, particle or bead or other solid phase such as hydroxylapatite or silica. This solid phase provides a simple means to remove the deprotection reagent after deprotection. However, primary amines such as ethylenediamine and ethanolamine can be used to deprotect the RNA when the RNA is either (i) attached to a solid support, or (ii) in solution.

Primary amines for use in this invention include; C1-C10 primary amines, C1-C10 hydroxylated primary amines, C1-C10 primary diamines, C1-C10 hydroxylated primary diamines, C1-C10 halogenated primary amines, C1-C10 halogenated primary diamines.

Very usefully, it has been found that primary amines such as ethylenediamine and ethanolamine not only serve as useful deprotection reagents but they also reduce the amount

of non-desired proteins that bind to hydroxylapatite and silica during nucleic acid purification. Hydroxylapatite binds to charged biomolecules by a combination of its positively charged calcium ions, negatively charged phosphate ions and hydroxyl groups. Although nucleic acids such as RNA and DNA carry a strong negative charge allowing binding to the hydroxylapatite calcium ions, so also do many proteins. Furthermore, many proteins are also positvely charged so that they bind to the phosphate groups of the hydroxylapatite. Frequently, the nucleic acid is in very low concentration compared with the contaminating proteins, lipids and carbohydrates present in the cell or biological sample such as blood, serum etc. As a result it can be difficult to purify the nucleic acid away from the contaminants using hydroxylapatite. Most methods have relied on using differential elution to separate the nucleic acids from the contaminants, however, such preparations are either low in the desired nucleic acid or contain significant amounts of the non desired contaminant. The contaminant may reduce the efficiency of downstream applications of the nucleic acid such as reverse transcription, PCR, hybridisation or even lead to its degradation if significant amounts of nucleases are eluted with the nucleic acid analyte.

Therefore, inhibiting proteins from binding to the hydroxylapatite whilst still allowing deprotection and nucleic aicd binding to occur is highly advantageous. Purification of nucleic acids, in particular RNA from serum and plasma, has been found to be improved by the addition of the deprotection amine reagent to the chemical modification reaction bearing the acylated RNA. For the purification of modified RNA from biological samples such as blood plasma without deprotection occuring but still inhibiting proteins from binding the purification matrix such as silica or hydroxylapatite can be accomplished by using secondary and tertiary amines. Secondary and tertiary amines have been found to inhibit proteins from binding to silica and hydroxylapatite but do not lead to deprotection of the RNA. Therefore, in this example, modified RNA can be purified from complex biological samples by the addition of a secondary or tertiary amine to the reaction before, or at the same time as the purification matrix is added to the mixture. The modified RNA can then be purified and eluted from the matrix in its protected form. The modified RNA can also be stored and archived in its protected form and deprotected using a primary amine immediately prior to use, for example for hybridisation or RT-PCR.

Likewise, DNA can be separated from contaminating proteins by the use of primary, secondary and tertiary amines in conjunction with the solid phase matrix such as silica or hydroxylapatite beads. The purpose of the amine in this case is not to remove acetyl groups from the DNA but to inhibit the binding of proteins to the purification matrix.

Secondary amines for use in this invention include ; C1-C10 secondary amines, C1-C10 hydroxylated secondary amines, C1-C10 secondary diamines, C1-C10 hydroxylated secondary diamines, C1-C10 halogenated secondary amines, C1-C10 halogenated secondary diamines.

Tertiary amines for use in this invention include ; C1-C10 tertiary amines, C1-C10 hydroxylated tertiary amines, C1-C10 tertiary diamines, C1-C10 hydroxylated tertiary diamines, C1-C10 halogenated tertiary amines, C1-C10 halogenated tertiary diamines.

Modified RNA can be deprotected at any one of three points during purification (i) at the same time as binding to the solid phase purification matrix as described in example 1 below, (ii) after the modified RNA has been been bound to the solid phase purification matrix, but before elution, and (iii) after elution from the solid phase purification matrix. The advantage of (i) is that the modified RNA is deprotected and proteins removed at the same time, the advantage of (ii) is that the deprotected RNA can be readily removed from the deprotection reagent by means of the solid phase it is bound to, whilst the advantage of (iii) is that the RNA is purified in its modified and therefore protected form. However, with method (iii) there is a necessity to separate the deprotected RNA from the deprotection reagent. This can be achieved by filtration using a Centricon device (Millipore, USA) according to maufacturer's instructions, precipitation using alcohol or dialysis. Alternatively, the RNA can be separated by evaporating the deprotection reagent away using reduced pressure or increased temperature. In this case, a deprotection reagent with a decreased boiling point is preferable to one with a high boiling point. Suitable primary amine reagents with a reduced boiling point are propylamine (bp 47°C) or butylamine (bp 76°C). Alternatively, the deprotected RNA can be removed from the deprotection reagent by binding it onto a solid phase deprotection reagent such as hydroxylapatite, washing the beads using 70% ethanol to remove excess deprotection reagent, and then eluting the RNA using phosphate or a chelator.

The use of magnetic or paramagnetic preparations of hydroxylapatite or even silica is preferred because of the ease of handling, washing and the large surface area of particles compared with other forms of the solid phase such as a membrane.

Example 1

Purification of RNA from human blood plasma

Various types of RNA can be purified from plasma such as the clinically important viruses HCV and HIV. The virus capsids are disrupted in the presence of 1-methylimidazole, tetrahydrofuran (THF) and an acylating reagent such as acetic anhydride. This mixture also leads to the disruption of the nucleoprotein complex and consequent release of the RNA which can then be chemically modified and stabilised.

Stabilisation of the RNA analyte : To 200µl of human plasma (EDTA coagulation inhibitor) or serum in a 15ml screw top polypropylene centrifuge tube (Falcon, USA) was added 50µl of 1-methylimidazole, the mixture was mixed briefly and then 600µl of a mixture of tetrahydrofuran and acetic anhydride (2 :1 vol/vol) was added and mixed by gentle pipetting with a 1ml pipette tip. A second addition of 600µl of tetrahydrofuran and acetic anhydride (2 :1 vol/vol) was added within 1 minute of the first addition and the solution mixed again. Other types of acylating reagents such as propionic, butanoic, pentanoic, heptanoic or benzoic anhydrides can be used as alternatives or in conjunction with acetic anhydride. Acylation reagents and methods for chemically modifying RNA have been described (WO/01/94626 and WO/00/75302)

Following addition of the acylation reagent, an internal control such as one composed of RNA (e.g. 8µl of the HCV Internal Control, version 2.0, Roche Diagnostics, USA) or DNA or even a bacteriophage (Armoured RNA, Hepatitis Virus Control, Ambion RNA Diagnostics, USA) may be added. Following an incubation for 10 minutes at 37°C, the solution containing the stabilised RNA can be stored for prolonged periods at up to 37°C. The RNA can also be transported and handled in its protected form. Alternatively, the RNA can be purified immediately following the 10 minute incubation at 37°C as described below.

It has been found that with human plasma samples such as those originating from blood donations, on addition of tetrahydrofuran and acetic anhydride, the reaction turns bright fluorescent yellow, serving as a useful indicator that the reaction was successful. On standing for 10 minutes the reaction turns brown.

Processing of the stabilised RNA : Following stabilisation of the RNA during which the RNA sample can be either stored, transported or processed immediately (following the 10 minute incubation at 37°C) the RNA is separated from the reaction and contaminants by differential binding to a solid support such as silica or hydroxylapatite in the presence of an organic amine. Alternatively, but less preferably the RNA can be purified by precipitation, dialysis or spin column filtration. The RNA can also be spotted and immobilised directly onto a solid support such as nylon (Hybond N+, AmershamPharmacia Biotech, UK) and analysed by hybridisation with a labelled complementary probe.

It has been found that primary amines such as ethylenediamine or ethanolamine are particularly suited to reducing contamination by protein binding to the solid phase used to bind and purify the nucleic acid, and in the case of RNA, also leads to the cleavage of the protecting acetyl group from the 2'-OH position of the RNA. The primary amine therefore has two useful properties (i) reducing protein contamination and, (ii) deprotecting the RNA without leading to consequent phosphodiester cleavage. The use of ethylenediamine and ethanolamine for the deprotection of oligonucleotides has been described (Miller, P.S. et al. (1986) Biochem. 25:5092; Hogrefe et al., Nucleic Acids Res (1994) 22:5492 ; Polushin, N. (1994) Nucleic Acids Res. 22 :639; Polushin, N. (1991) Nucleic Acids Symp Ser. 24 :49).

To 1.45ml of the reaction containing the desired RNA analyte is added 1.4ml of ice cold 1-methylimidazole and the solution mixed by gentle pipetting. Then three separate aliquots of 200µl of a prepared solution of 1ml of 1-methylimidazole, 1ml of either ethanolamine or preferably ethylenediamine containing 50µl (40mg/ml) of phosphate treated hydroxylapatite Type I (Chemicell, Germany) are added and the reaction incubated for 2 minutes at 25°C, before the remaining 1.45ml of the 1-methylimidazole, primary amine and beads are added and mixed. The entire mixture is then slowly mixed using an end-over-end wheel for 10 minutes at 25°C to allow deprotection of the RNA and binding to the beads. However, agitating the mixture is not essential. The addition of the amine leads to an exothermic

reaction, and the amine is therefore most easily added to the RNA containing reaction diluted into the buffer 1-methylimidazole. Alternatively, silica may be used in the place of hydroxylapatite to bind the nucleic acid.

Phosphate treatment of the beads is as follows ; to 1ml of hydroxylapatite Type I (50mg/ml) was added 2ml of 0.2M sodium phosphate (pH 7) and the beads are briefly mixed by inverting the tube. The beads are then collected with a magnet and the liquid discarded, the bead pellet is then resuspended in 2ml of water, briefly mixed, the beads collected and the water wash repeated once more, the beads are then resuspnded in 1ml of water. It has been found that hydroxylapatite beads treated in this way bind less protein and the RNA is more easily eluted than the non-phosphate treated beads.

The beads are then collected from the reaction using a magnetic stand (Dynal, Norway) and the liquid discarded by pouring it away from the bead pellet. The beads are then resuspended in 1ml of 70% methanol/ethanol (1 :1 vol/vol) and transferred to a fresh 2ml polypropylene centrifuge tube and collected using a magnetic stand (Promega, USA). The beads are washed two more times in 1ml of 70% methanol/ethanol and then washed three times in 100µl of water. It is important not to touch the beads with the pipette tip when they are in aqueous suspension because they tend to stick to plastic resulting in sample loss. The RNA is now ready for storage, transport or can be immediately eluted from the hydroxylapatite beads.

The nucleic acid can be released from the hydroxylapatite bead by a variety of means including the use of phosphate containing solutions or a divalent metal ion chelator such as EGTA. The mechanism of nucleic acid elution most probably involves a competition between the nucleic acid phosphate groups and the hydroxylapatite calcium atoms. It has been found that nucleotide triphosphate groups and metal ion chelators are particularly effective at displacing nucleic acids from the hydroxylapatite.

To the nucleic acid hydroxylapatite bead mixture (approximate volume 50-100µl) is added 50µl of a nucleotide solution, the beads agitated by means of a magnetic stirrer set on the program 0.5 second step, (MCB1200, Dexter Magnetics, UK) for 2 minutes at 25°C, the beads collected using the magnet, the liquid collected and then the process repeated 3 more

times and the liquid containing the nucleic acid pooled. The nucleotide solution can be a 5mM dNTP solution such as dATP, dCTP, dGTP, TTP, dUTP or a 5mM solution of ribose NTP such as rATP, rCTP, rGTP, rUTP or a 50mM solution of dNDP or rNDP, dNMP or rNMP or even inorganic pyrophosphate (sodium phosphate) as described (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH). It has been found that as the nucleic acid is displaced from the hydroxylapatite, the total nucleotide concentration is reduced indicating it is binding to the hydroxylapatite in place of the eluted nucleic acid. This is important when calculating the final nucleotide concentration for downstream analytical procedures such as RT-PCR.

Alternatively, and preferably, the nucleic acid can be eluted from the hydroxylapatite using calcium ion chelators such as CDTA (trans-1,2-Diaminocyclohexane-N,N,N`,N`-tetraacetic acid), EDTA (Etylenediamine tetraacetic acid), EGTA (Etylenenglycol-O,O`-bis(2-aminoethyl)-N,N,N`,N`-tetraacetic acid), DTPA (Diethylenetriamine pentaacetic acid), HEDTA (N-(2-Hydorxyethyl)ethylenediamine-N,N,N`-triacetic acid), NTA (Nitrilotriacetic acid), TTHA (Triethylenetetramine-N,N,N`,N``,N```,N```-hexaacetic acid), Dimethyl-BAPTA (Molecular Probes, USA) or BAPTA (Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid). To the nucleic acid hydroxylapatite bead mixture (approximate volume 50-100µl) is added 50µl of a 10mM chelator solution, the beads agitated by means of a magnetic stirrer set on the program 0.5 second step, (MCB1200, Dexter Magnetics, UK) for 2 minutes at 25°C, the beads collected using the magnet, the liquid collected and then the process repeated 7 more times and the liquid containing the nucleic acid pooled. It has been found that 75% of the nucleic acid is eluted between the 5th to the 8th elution (200-400µl) of a 10mM solution of EGTA. By comparison, it has also been found that DNA is eluted almost equally into the 1st to the 7th 50µl elution volume as determined by both 32P labelled DNA tracer studies and PCR amplification of the eluted DNA.

The eluted RNA and the material used to elute it from the hydroxylapatite can either be used directly in an analytical procedure such as hybridisation, or in an enzymatic assay such as reverse transcription-PCR. Whilst phosphate containing elution solutions work efficiently to elute nucleic acids from hydroxylapatite, they are not ideal because excess phosphate tends to interact and therefore reduce the final concentration of metal ions in enzymatic reactions leading to reduced yields. Therefore it is preferred to use metal ion chelators such as EGTA

which have higher specificity for certain types of divalent metal ions. It has been found that when 10mM EGTA is used to elute the nucleic acid from the hydroxylapatite, the final EGTA concentration is approximately 4-5mM. EGTA has a lower stability constant (logK 5.21) for magnesium ions than EDTA (logK 8.69), therefore it has been found that the presence of at least 8mM (final EGTA concentration) in a RT-PCR reaction does not detectably alter the amplicon yield. Therefore for assays employing magnesium as a divalent metal cation at, for example 2mM or more, such as MULV, AMV and Taq DNA polymerases, EGTA is a good choice for eluting the nucleic acid.

However, it has also been found that EGTA/ nucleic acid solutions tend to chelate manganese ions such as those solutions employed in commercial diagnostic RT-PCR assays for example the Amplicor HCV Test, version 2.0 (Roche Diagnostics, USA) leading to reduced amplification yield when too much of the EGTA/ nucleic acid solution is added. It has been found for example that the addition of 6µl or more of the EGTA/ nucleic acid solution to a standard 100µl Amplicor HCV Test, version 2.0 leads to reduced OD660nm readings indicating that the amplification efficiency is reduced.

Where the assay employs manganese ions, there are several practical solutions to the problem of chelation and therefore inhibition of the amplification. Firstly the chelator can be removed using a differential binding process whereby the chelator molecule such as EGTA can diffuse through a semi-permeable material / barrier such as a gel, membrane, polymer or pore and bind to a chelator binding material such as hydroxylapatite. It has been found that suitable methods for removing small chelators from a nucleic acid solution are (i) mixing hydroxylapatite beads with 0.2-0.8% molten agarose or preferably polymerising acrylamide with hydroxylapatite and allowing it to solidify thereby entombing the hydroxylapatite. The EGTA/ nucleic acid solution is then placed in contact with the gel and the EGTA (or other chelator) allowed to diffuse into the gel, whilst the gel prevents the larger RNA molecules from contacting the beads and therefore from being lost. It has been found that 20 minutes is sufficient to remove 90% of the EGTA from a 50µl volume of 5mM EGTA solution at ambient temperature when placed in contact with a 1cm2 area of 100µl of the gel containing 0.5mg hydroxylapatite (type I, Chemicell, Germany). The remaining liquid containing the nucleic acid is then added to the assay. Alternatively, the hydroxylapatite beads can be coated in a polymer such as a heparin, cellulose, starch or dextran to provide a semi-

permeable barrier to the nucleic acid whilst still allowing the chelator molecules to bind the hydroxylapatite. A 200µl volume of the polymer solution (10%) is conveniently added to 0.5mg hydroxylapatite (type I, Chemicell, Germany) beads and allowing the solution to dry completely at 90°C for 1 hour.

Another method (ii) for removing the chelator is to filter the nucleic acid / chelator solution. Although many filtration methods exist for selectively removing contaminants from nucleic acids, such as size exclusion chromatography and silica membranes, a preferred method is to use centrifugal or pressure filter devices, also known as ultrafiltration such as those known as Centricon, Centriprep, Centriplus and Centricon Plus® manufactured by Millipore (USA). In particular it has been found that Microcon centrifugal devices are well suited for this purpose. These devices use regenerated cellulose with various pore sizes with molecular weight cut offs from 3 000-100 000 daltons. The preferred size is 50 000 daltons molecular weight cut off (Part No. 42416, Millipore, USA) so that nucleic acids more than 200 nucleotides in length are retained by the membrane, whilst the chelator such as EGTA passes through the membrane and are discarded. The nucleic acid can be removed from the hydroxylapatite using a chelator, a phosphate containing solution, or a calcium binding salt. An advantage of the filtration method is that not only are contaminants removed but the nucleic acid is concentrated. This is particularly advantageous when the elution solution containing the nucleic acid is more than 100µl because the final filtered and concentrated volume can be 10µl so that the entire sample can be added to the analytical test. For example with the HCV Amplicor test v2.0 (Roche Diagnostics, USA) the maximum volume that may be added to each test is 50µl, whilst the eluted nucleic acid volume may be as much as 0.5ml. The filtration therefore serves two purposes ; to remove the contaminants and to concentrate the analyte.

The preferred method of filtration is as follows. Using 10mM EGTA as the elution solution, the first 400µl of the elution from the hydroxylapatite beads is collected and pooled. The elution can either be 8 separate elutions of 50µl with 2 minute elution steps using mixing as described above (MCB1200, Dexter Magnetics, USA) or 1 elution with 400µl of 10mM EGTA with a ten minute elution with mixing. It is also possible to add a smaller volume of more concentrated elution solution such as 200µl of 20mM EGTA and mixing for 10

minutes at room-temperature. In any case, the final elution volume is conveniently no more than 400 µl which is the maximum volume accomodated by the Microcon filtration device (Part No. 42416, Millipore, USA). The device is then centrifuged at 12 000g for 20 minutes (until dryness), 400µl of water added and the device spun again at 12 000g for 20 minutes (until dryness). Then 25-50µl of water is added to the device to recuperate the nucleic acid the cup inverted in a 2ml fresh tube and centrifuged for 10 seconds at 2500g. The nucleic acid can then be used in the assay. Proteins that are larger than 50 000 daltons are also retained with the nucleic acid, however, it has been found that no detectable inhibition occured during RT-PCR (HCV Amplicor v2.0, Roche Diagnostics, USA) with RNA prepared in this manner from blood plasma. Alternatively the nucleic acid can be eluted from the hydroxylapatite using 400ml of 100-500mM Sodium Phosphate (pH7) and mixing for 10 minutes at room temperature. The phosphate/ nucleic acid solution can then be filtered using a Microcon filtration device as described above.

Yet another method (iii) for relieving the inhibitory effects of the chelator and in particular the EGTA solution is to 'neutralise' the capacity of the chelator to bind the manganese or magnesium. This can be accomplished in a number of ways. The first method is to add a metal ion that has a higher stability constant for the chelator than the metal ion necessary for the assay. Unfortunately Mn ions have a particularly high stability constant (12.3) with EGTA compared with Mg ions (5.21). Therefore it is preferable to choose metal ions with stability constants higher than Mn (12.3) so that the added metal ion competes effectively with the Mn for the chelator. Suitable metal ions are Fe(III) (20.3), Cu (II) (17.8), Co (II) (12.30) and Zn (II) (14.5). These metal ions can be added to the chelator containing solution as a salt such as the chloride or acetate. Although other metal ions also have high stability constants for EGTA, such as Ni, Hg and Cd, their toxicity would preclude their routine use. It has been found that adding 3.5-7mM final concentration of CoCl2 to a 100µl standard HCV Amplicor (Roche Diagnostics, USA) reaction containing 25µl of RNA eluted from the hydroxylapatite beads using 10mM EGTA effectively removes the inhibitory effects of the EGTA allowing amplification of the HCV analyte RNA. The most preferable CoCl2 concentrations are 4.5mM and 6mM. Alternatively, CuCl2 or FeCl3 can also be used in the range 3.5-7mM but are less effective.

It is also possible to use an additional amount of Mn in the analytical procedure to replace the Mn bound to the chelator. It has been found that adding 1-3mM, or preferably 1.5mM Manganese acetate to a 100µl standard HCV Amplicor reaction containing 25µl of RNA eluted from the hydroxylapatite beads using 10mM EGTA effectively removes the inhibitory effects of the EGTA allowing amplification of the HCV analyte RNA. Therefore it is not strictly necessary that the metal ion has a stability constant higher than Mn in order to overcome the inhibition. However, 4.5mM CoCl2 is preferred in the HCV Amplicor test as described above.

If silica particles or membranes are used for capturing the nucleic acid, then an equal weight of silica such as Qiaex II (Qiagen, Germany) or Magnisil (Promega, USA) is added in the place of hydroxylapatite beads. In this case the silica beads, following the deprotection reaction with the primary amine are washed in four rinses of 1ml of 70% ethanol and then the nucleic acid is eluted in 200µl of water following incubation for 10 minutes at 37°C.

Example 2

Purification of DNA from human blood plasma

Various types of DNA can be purified from plasma such as the clinically important virus HBV or bacteria. The viral or bacterial particles are disrupted in the presence of 1-methylimidazole, tetrahydrofuran (THF) and an acylating reagent such as acetic anhydride. This mixture also leads to the disruption of the nucleoprotein complex and consequent release of the DNA which can then be chemically modified and stabilised.

Methods for purifying DNA are substantially similar to methods for purifying RNA as set out in Example 1 above. However, unlike RNA, ds DNA (300bp) tends to elute from the first to the 7th addition of 50µl of 10mM EGTA, therefore all fractions of the elution should be kept.

Example 3

Purification of DNA and RNA simultaneously from human plasma

Both DNA and RNA can be purified from plasma at the same time, allowing simultaneous testing of both clinically important RNA sources such as HCV and HIV as well as DNA sources such as HBV from the same eluted nucleic acid sample. The viral or bacterial

particles are disrupted in the presence of 1-methylimidazole, tetrahydrofuran (THF) and an acylating reagent such as acetic anhydride. This mixture also leads to the disruption of the nucleoprotein complex and consequent release of the DNA which can then be chemically modified and stabilised.

Methods for purifying DNA and RNA are substantially similar to methods set out in Example 1 and 2 above.

Example 4

Purification of DNA and/ or RNA from whole human blood

If present, both DNA and RNA can be purified from whole blood at the same time, allowing testing of both clinically important RNA sources such as HCV and HIV as well as DNA sources such as HBV from the same eluted nucleic acid sample.

Methods for purifying DNA and/ or RNA are substantially similar to Example 1 above, except that 200µl, instead of 50µl of 1-methylimidazole is added to 200µl of blood. It has been found that the addition of 200µl, instead of 50µl of 1-methylimidazole helps to solubilise the cellular components present in the blood as the erythrocytes and white blood cells. Although it has been found that 50µl of 1-methylimidazole also works well, the mixture of 1-methylimidazole, tetrahydrofuran and acetic anhydride tends to form large clumps which only dissolve once the deprotection reagent (e.g. ethylenediamine) is added.

Example 5

Purification of DNA and/ or RNA from cells

Both DNA and RNA can be purified from cells at the same time, allowing purification of both DNA and cellular RNA such as rRNA, tRNA and mRNA.

Methods for purifying DNA and/ or RNA are substantially similar to Example 1 above, except that 200µl, instead of 50µl, of 1-methylimidazole is added to 200µl of a centrifuge collected pellet (3 000g x 10 minutes) of 1 million tissue culture cells. It has been found that the addition of 200µl, instead of 50µl of 1-methylimidazole helps to solubilise the cellular components present. Although it has been found that 50µl of 1-methylimidazole also works

well, the mixture of 1-methylimidazole, tetrahydrofuran and acetic anhydride tends to form large clumps which only dissolve once the deprotection reagent (e.g. ethylenediamine) is added.

Alternatively, 50mg of tissue or organ sample can be disrupted using a dounce homegeniser or sonicator in the presence of 200ml of 1-methylimidazole, then the mixture immediately added to 600µl of THF/ acetic anhydride (2 :1 vol :vol), mixed before a second addition of 600µl of THF/ acetic anhydride (2 :1 vol :vol). Following a 10 minute incubation at 37°C the RNA is purified identically as for Example 1 starting at the addition of 1.4ml of 1-methylimidazole.

Example 6

Purification of DNA from human faeces or urine

The method is as described as for example 1 except that the 200µl of plasma is replaced by 200µl of urine or 200µl of faeces diluted in water to 10%.

Example 7

Deprotection of acetyl modified RNA using gaseous ammonia

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of acetic anhydride was mixed into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded. The beads were washed once with 95% ethanol and allowed to air dry for 5 minutes at 25°C.

Deprotection of the modified RNA. The open tube containing the bead-modified RNA mixture was placed in a 50ml screw top polypropylene tube and a pipe inserted into a hole through the cap of the 50ml tube. 100ml of 38% ammonium hydroxide was heated to 50°C in a side arm flask, the ammonia vapour was allowed to exit the flask and enter a separate

side arm glass flask to allow condensation of any water vapour. The dried ammoni gas was then allowed to enter the 50 ml tube containing the beads by means of a flexible plastic pipe. The screw cap of the 50ml tube was loosely closed allowing ammonia gas to enter the tube and then exit to be replaced by fresh ammonia.

The acetylated RNA on the beads was subjected to the ammonia for 5-60minutes, after which the beads were washed once in water and then eluted with 10mM EDTA or EGTA (hydroxylapatite) or the deprotected RNA was eluted directly into 50µl water (silica beads). It was found tht the time for deprotection varied according to the amount of ammonia produced during heating, 60 minutes usually being sufficient to remove substantially all acetyl groups from the RNA. The deprotected RNA can then be used for various downstream applications such as RT-PCR and hybridisation.

Example 8

Deprotection of acetyl modified RNA using ethylenediamine

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of acetic anhydride was mixed into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded.

To the wet beads was added 50-500µl of ethylenediamine (Fluka cat. No. 03550, France) and the beads stirred briefly and incubated for 1-60 minutes at 25°C. The beads were then collected with a magnet and the liquid discarded. The beads were washed twice with 200µl of 70% ethanol/methanol (1 :1) once with water and then the deprotected RNA was eluted into 100µl of 10mM EDTA or EGTA by incubating for 10 minutes at 25°C. It was found that 1 minute with 300µl of ethylenediamine was not sufficient to fully deprotect the RNA as assayed using an *in vitro* transcript labelled with 32P and a 5% acrylamide sequencing gel. The rate of migration is proportional to the amount of deprotection and sequencing gels

serve as a useful measure of the amount of deprotection that has occured. After 5 minutes the RNA was over 90% deprotected and after 15 minutes the RNA was completely deprotected. Increasing the deprotection time to 60 minutes at 25°C did not lead to any detectable RNA degradation.

Example 9

Deprotection of acetyl modified RNA using ethanolamine

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of acetic anhydride was mixed into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded.

To the beads that were not dried from the previous protection reaction, was added 50-500µl of ethanolamine (Fluka cat. No. 02400, France) and the beads stirred briefly and incubated for 1-60 minutes at 25°C. The beads were then collected with a magnet and the liquid discarded. The hydroxylapatite beads were washed twice with 200µl of 70% ethanol/methanol (1 :1), once with water and then the deprotected RNA was eluted into 100µl of 10mM EDTA or EGTA by incubating for 10 minutes at 25°C. The silica beads were washed twice in 200µl of Wash solution PE (Qiagen, Germany) and the deprotected RNA eluted in 100µl of water.

It was found that 1 minute with 300µl of ethylenediamine was not sufficient to fully deprotect the RNA as assayed using an *in vitro* transcript labelled with 32P and a 5% acrylamide sequencing gel. The rate of migration is proportional to the amount of deprotection and sequencing gels serve as a useful measure of the amount of deprotection that has occured. After 5 minutes the RNA was over 70% deprotected and after 15 minutes the RNA was was over 90% deprotected. Complete deprotection occured after 30 minutes at 25°C. Increasing the deprotection time to 60 minutes at 25°C led to detectable degradation

of a 1700 nucleotide RNA molecule but relatively little degradation of a 250 nucleotide molecule as determined by sequencing gel analysis. Therefore deprotection with ethanolamine was slightly slower than with ethylelenediamine and also led to more RNA degradation. However, this may have been at least in part due to the purity of the ethanolamine.

Example 10

Deprotection of acetyl modified RNA using mixtures of ethanolamine and ethylenediamine

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of acetic anhydride was mixed into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded.

To the wet beads was added 50-500µl of a mixture of ethanolamine and ethylenediamine (1 :1), (Fluka, France) and the beads stirred briefly and incubated for 1-60 minutes at 25°C. The beads were then collected with a magnet and the liquid discarded. The hydroxylapatite beads were washed twice with 200µl of 70% ethanol/methanol (1 :1) once with water and then the deprotected RNA was eluted into 100µl of 10mM EDTA or EGTA by incubating for 10 minutes at 25°C. The silica beads were washed twice in 200µl of Wash solution PE (Qiagen, Germany) and the deprotected RNA eluted in 100µl of water.

Example 11

Deprotection of acetyl modified RNA using ethanolamine or ethylenediamine at increased temperatures

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of acetic anhydride was mixed

into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded.

To the wet beads was added 100µl of ethylenediamine or ethanolamine and the beads stirred briefly and incubated for 20 minutes at 37, 45 and 55°C. The beads were then collected with a magnet and the liquid discarded. The beads were washed twice with 200µl of 70% ethanol/methanol (1 :1) once with water and then the deprotected RNA was eluted into 100µl of 10mM EDTA or EGTA by incubating for 10 minutes at 25°C.

The amount of deprotection of the RNA as assayed using an *in vitro* transcript labelled with 32P and a 5% acrylamide sequencing gel. The rate of migration is proportional to the amount of deprotection and sequencing gels serve as a useful measure of the amount of deprotection that has occured. It was found that with ethanolamine at 55, 45 or 37°C there was significant degradation of the RNA, whilst with ethylenediamine at 55°C there was limited degradation, both deprotection at 45 or 37°C did not lead to detectable RNA degradation. Thgerefore deprotection with ethanolamine is best achived at 25°C whilst with ethylenediamine, deprotection can be carried out up to 45°C.

Example 12

Deprotection of acetyl modified RNA using ethanolamine or ethylenediamine in alcohol

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of acetic anhydride was mixed into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded.

To the wet beads was added 100µl of ethylenediamine or ethanolamine and 100µl of methanol/ethanol (1:1) and the beads stirred briefly and incubated for 20 minutes at 37, 45 and 55°C. The beads were then collected with a magnet and the liquid discarded. The beads were washed twice with 200µl of 70% ethanol/methanol (1:1) once with water and then the deprotected RNA was eluted into 100µl of 10mM EDTA or EGTA by incubating for 10 minutes at 25°C.

It was found that the addition of alcohol was not beneficial to the deprotection reaction, indeed the rate of deprotection was reduced whilst the amount of RNA degradation was increased.

Example 13

Deprotection of acetyl modified RNA using ethanolamine or ethylenediamine with a strong alkali

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of acetic anhydride was mixed into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded.

To the wet beads was added 100µl of ethanolamine plus either 10µl of 10% ammonium hydroxide, 10µl of 10mM NaOH or 10µl of 50mM NaOH and the beads stirred briefly and incubated for 20 minutes at 37°C. The beads were then collected with a magnet and the liquid discarded. The beads were washed twice with 200µl of 70% ethanol/methanol (1 :1) once with water and then the deprotected RNA was eluted into 100µl of 10mM EDTA or EGTA by incubating for 10 minutes at 25°C.

It was found that the addition of either ammonium hydroxide or NaOH did not increase the amount of deprotection compared with ethanolamine alone bu did increase the amount of RNA degradation.

Example 14

Deprotection of acetyl modified RNA using ethanolamine and ethylenediamine in the presence of water

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of acetic anhydride was mixed into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded.

To the wet beads was added 50µl of ethanolamine, 10µl of water and 50µl of methanol and the beads stirred briefly and incubated for 5 minutes at 25°C. The beads were then collected with a magnet and the liquid discarded. The beads were washed twice with 200µl of 70% ethanol/methanol (1 :1) once with water and then the deprotected RNA was eluted into 100µl of 10mM EDTA or EGTA by incubating for 10 minutes at 25°C.

It was found that the addition of water did not alter the amount of deprotection compared with ethanolamine and alcohol alone and did not increase the amount of RNA degradation. It is therefore not essential that water be removed from solutions and the beads prior to deprotection.

Example 15

Alternative means to remove RNA from hydroxylapatite beads

It has been found that either protected or deprotected RNA can be removed from hydroxylapatite beads by simply loading the bead-RNA complex into either a well of a 0.5 X

TAE agarose gel or a well of a 1 X TBE sequencing gel and applying an electric field through the well containing the beads. The protected or deprotected RNA readily dissociates from the hydroxylapatite beads and electrophoresis into the gel where it can be either collected or analysed by means for example of EtBr or a radioactive label. This is a very convenient means to detach RNA from hydroxylapatite.

Protected or deprotected RNA may also be separated from hydroxylapatite by inserting 2 wires (anode and cathode) without them touching into a tube containing beads in 100µl of water or buffer and applying a low voltage such as 5-50V for 5 minutes. The RNA can be recuperated from the liquid phase.

Example 16

RT-PCR amplification of Deprotected RNA

Preparation of modified template RNA. To 100µl of a mixture of tetrahydrofuran/acetic anhydride (2 :1 vol/vol) was added 14µl of 1-methylimidazole and 2µg of BMV RNA (Promega, USA), the mixture stirred and incubated for 2 minutes at 25°C. To the reaction was added 60µl of 1-butanol and then 20µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) and mixed for 3 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA), washed once in 200µl of 70% methanol/ ethanol (1:1) and the liquid discarded.

To the wet beads was added either 100µl of ethanolamine or 100µl of ethylenediamine and the beads stirred briefly and incubated for 20 minutes at 37°C. The beads were then collected with a magnet and the liquid discarded. The beads were washed twice with 200µl of 70% ethanol/methanol (1 :1) once with water and then the deprotected RNA was eluted into 100µl of 10mM EGTA by incubating for 10 minutes at 25°C.

Reverse Transcription. 25 ng of the deprotected BMV RNA was added to a 20 µl reaction mixture containing the following final component concentrations: 200 mM Tris-HCl (pH 8.4 at 24°C), 75 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP's, 60 ng of oligonucleotide primer BMV R (GAGCCCCAGCGCACTCGGTC) and MULV RNase H^-

(Promega, cat no. M3682, USA). Water was used to bring the final volume to 20 µl. The reaction was allowed to proceed for 20 minutes at 37°C, 20 minutes at 42°C and 20 minutes at 50°C. PCR Amplification. The PCR was carried out in a final volume of 25µl with final concentration of 15mM Tris-HCl pH 8.8, 60mM KCl, 2.5mM $MgCl_2$, 400 µM of each dNTP, 10 pmol of each primer BMV F (CTATCACCAAGATGTCTTCG) and BMV R and 1 unit *Taq* DNA polymerase (Roche Molecular, France). To the PCR mix was added 2 µl of cDNA generated from the deprotected BMV RNA. Cycle parameters were 94°C x 8 sec, 58°C x 8 sec and 72°C x 15 sec for 30 cycles. The 250bp PCR products were visualised following gel electrophoresis and staining with EtBr.

Excellent amplification was observed with both ethylenediamine and ethanolamine deprotected RNA, indeed no significant differences could be seen in the yield of PCR product between protected-deprotected RNA compared with an untreated RNA control indicating that no substantial degradation of the RNA occured during deprotection.

Example 17

Hybridisation of Deprotected RNA

Preparation of modified template RNA. To 100µl of a mixture of tetrahydrofuran/acetic anhydride (2 :1 vol/vol) was added 14µl of 1-methylimidazole and 2µg of BMV RNA (Promega, USA), the mixture stirred and incubated for 2 minutes at 25°C. To the reaction was added 60µl of 1-butanol and then 20µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) and mixed for 3 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA), washed once in 200µl of 70% methanol/ ethanol (1:1) and the liquid discarded.

To the wet beads was added either 100µl of ethanolamine or 100µl of ethylenediamine and the beads stirred briefly and incubated for 20 minutes at 37°C. The beads were then collected with a magnet and the liquid discarded. The beads were washed twice with 200µl of 70% ethanol/methanol (1 :1) once with water and then the deprotected RNA was eluted into 100µl of 10mM EGTA by incubating for 10 minutes at 25°C.

Immobilisation of deprotected RNA. 100, 50 or 25 ng of the deprotected BMV RNA was added to a 20 µl reaction mixture containing the following final component concentrations: 200 mM Tris-HCl (pH 8.4 at 24°C), 75 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP's, 60 ng of oligonucleotide primer BMV R (GAGCCCCAGCGCACTCGGTC) and MULV RNase H^- (Promega, cat no. M3682, USA). Water was used to bring the final volume to 20 µl. The reaction was allowed to proceed for 20 minutes at 37°C, 20 minutes at 42°C and 20 minutes at 50°C. PCR Amplification. The PCR was carried out in a final volume of 25µl with final concentration of 15mM Tris-HCl pH 8.8, 60mM KCl, 2.5mM $MgCl_2$, 400 µM of each dNTP, 10 pmol of each primer BMV F (CTATCACCAAGATGTCTTCG) and BMV R and 1 unit *Taq* DNA polymerase (Roche Molecular, France). To the PCR mix was added 2 µl of cDNA generated from the deprotected BMV RNA. Cycle parameters were 94°C x 8 sec, 58°C x 8 sec and 72°C x 15 sec for 30 cycles. The 250bp PCR products were visualised following gel electrophoresis and staining with EtBr.

Excellent amplification was observed with both ethylenediamine and ethanolamine deprotected RNA, indeed no significant differences could be seen in the yield of PCR product between protected-deprotected RNA compared with an untreated RNA control indicating that no substantial degradation of the RNA occured during deprotection.

Example 18

Deprotection of propanoyl modified RNA using ethylenediamine

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of propionic anhydride (Fluka cat. No. 81942) was mixed into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded.

To the wet beads was added 50-500µl of ethylenediamine (Fluka cat. No. 03550, France) and the beads stirred briefly and incubated for 1-60 minutes at 25°C. The beads were then collected with a magnet and the liquid discarded. The beads were washed twice with 200µl of 70% ethanol/methanol (1:1) once with water and then the deprotected RNA was eluted into 100µl of 10mM EDTA or EGTA by incubating for 10 minutes at 25°C. It was found that 1 minute with 300µl of ethylenediamine was not sufficient to fully deprotect the RNA as assayed using an *in vitro* transcript labelled with 32P and a 5% acrylamide sequencing gel. The rate of migration is proportional to the amount of deprotection and sequencing gels serve as a useful measure of the amount of deprotection that has occured. After 5 minutes the RNA was over 90% deprotected and after 15 minutes the RNA was completely deprotected. Increasing the deprotection time to 60 minutes at 25°C did not lead to any detectable RNA degradation.

Example 19

Deprotection of acetyl modified RNA using polmer bound ethylenediamine

Ethylenediamine and several other types of primary amines are commercially available bound to a solid (polymer) support. Such primary amines are suitable for deprotecting acetylated RNA. They provide a convenient method for removing the deprotection reagent from the reaction once the deprotection is complete.

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of acetic anhydride (Fluka cat. No. 81942) was mixed into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded. The acetylated RNA on the hydroxylapatite beads were eluted into 200µl of 10mM EDTA or EGTA by incubating for 10 minutes at 25°C. The chelator/acetylated RNA solution (200µl) was then added to 100mg of ethylenediamine beads (Sigma-Alrdich Part No. 54,748,4, USA) and incubated for 1hr at 37°C. The beads

are conveniently removed by centrifugation (15 000rpm for 10 seconds) or filtration (Microcon device, Millipore, USA) leaving the deprotected RNA in solution.

Example 20

Deprotection of acetyl modified RNA using Propylenediamine

Preparation of modified RNA. To 40µl of tetrahydrofuran containing 16% 1-methylimidazole was added 100ng-1µg of RNA, then 2-4µl of acetic anhydride was mixed into the reaction and incubated for 10 minutes at 37°C. To the reaction was added 10µl of magnetic hydroxylapatite Type 1 (40mg/ml) (Chemicell GmbH, Berlin, Germany) or magnetic silica particles (« Magnisil », Promega, USA) and mixed for 10 minutes at 25°C. The beads were then collected using a magnetic stand (Promega, USA) and the liquid discarded.

To the beads that were not dried from the previos protection reaction, was added 50-500µl of propylenediamine (Fluka cat. No. 82250, France) and the beads stirred briefly and incubated for 1-60 minutes at 25-37°C. The beads were then collected with a magnet and the liquid discarded. The hydroxylapatite beads were washed twice with 200µl of 70% ethanol/methanol (1 :1), once with water and then the deprotected RNA was eluted into 100µl of 10mM EDTA or EGTA by incubating for 10 minutes at 25°C. The silica beads were washed twice in 200µl of Wash solution PE (Qiagen, Germany) and the deprotected RNA eluted in 100µl of water.